TABLE 1. Prostate gland formation in the recombinates of the urogenital sinus epithelium of *Tfm* or wild-type mice with the urogenital sinus mesenchyme of normal rats

Recombinates (epithelium/ mesenchyme)	Culture period (weeks)	Prostate glands	Seminal vesicles	Coagulating glands	Urethral glands
	4	15/16	4/16	3/16	9/16
Tfm-mouse/	6	5/5	1/5	0/5	4/5
Rat	8	4/4	1/4	0/4	4/4
Wild-mouse/	4	11/11	6/11	1/11	4/11
Rat					

TABLE 2. Alkaline phosphatase and nonspecific esterase activities in the prostatic glands induced in the explants

Recombinates (epithelium/ mesenchyme)	Culture period (weeks)	Number of explants	Tissues	Alkaline phosphatase activity	Nonspecific esterase activity
	4	9	E	−	+∼+++
Tfm-mouse/			M	++∼+++	−
Rat	8	4	E	NE	−
			M	NE	−
Wild-mouse/	4	5	E	−	+∼+++
Rat			M	++∼++	−

NE: not examined, −: negative, +: weak, ++: strong, +++: very strong.
E: epithelium, M: lamina propria.

rived from the sinus epithelium of *Tfm* mice. The results are summarized in Table 3. In control explants, the epithelial nuclei of the glands were labelled strongly and the nuclei of the lamina propria moderately (Fig. 10). In contrast, the nuclei of most of the glandular epithelium derived from the *Tfm* mice were negative but the intensity of labelling in the lamina propria was similar to that in the control explants (Fig. 11). In competition experiments, unlabelled testosterone abolished nuclear labelling completely. The results show that in the recombinates with *Tfm* epithelium the androgen receptors were localized in the lamina propria of the prostate, that few or no receptors were induced in the *Tfm* epithelium, and that practically no androgen was transferred into the epithelium from the lamina propria. Nevertheless prostatic gland formation took place in the recombinates of the androgen-receptor defective epithelium with the normal mesenchyme.

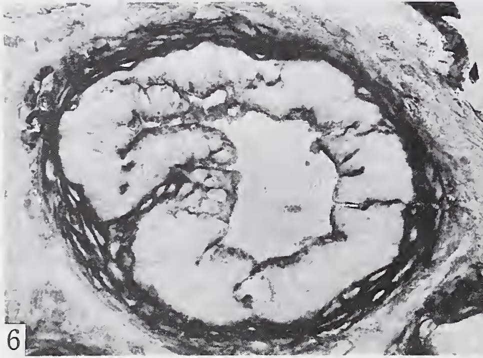
DISCUSSION

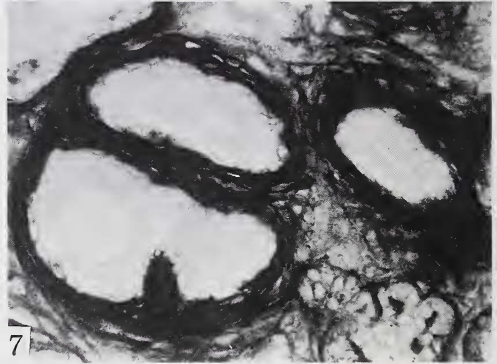
The results presented here show that the sinus epithelium of *Tfm* mice forms functional prostatic glands under the influence of the sinus mesenchyme of wild type rats in the presence of androgens, that the nuclei of the lamina propria

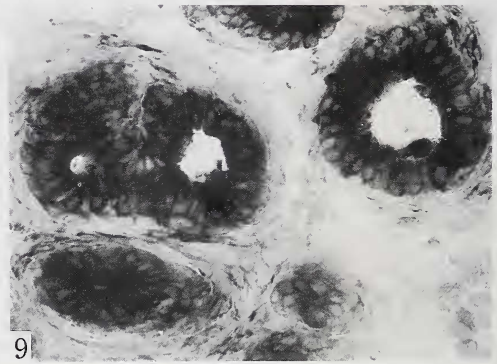
FIGS. 1 and 2. Histology of prostatic glands induced in the recombinates of the sinus epithelium of the wild-type (Fig. 1) or of the *Tfm*-type (Fig. 2) and the sinus mesenchyme of the wild-type. The culture period was 4 weeks. Magnification ×760. Haematoxylin-eosin staining.

FIG. 3. Hoechst staining of the prostatic glands induced in the recombinates of the mouse epithelium of *Tfm*-type and the rat mesenchyme. All the epithelial cells were mouse cells.

FIGS. 4 and 5. Epithelial cells of the prostatic glands induced in the recombinates of the *Tfm*-epithelium and the rat mesenchyme (Fig. 4) and those of the ventral prostate of a mouse of 4 weeks old (Fig. 5). Magnification ×5,000.







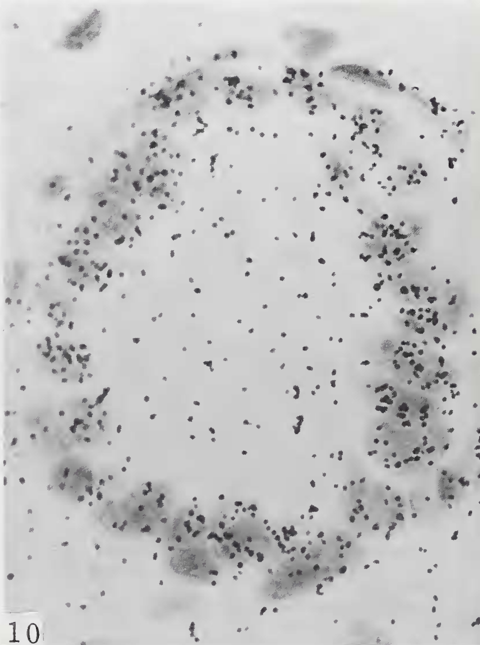


TABLE 3. Incorporation of [^{3}H]testosterone into the nuclei of the epithelium and mesenchyme of the induced prostatic glands

Recombinates (epithelium/ mesenchyme)	Culture period (weeks)	Number of explants	Tissues	Nuclear androgen incorporation
Tfm-mouse/	4	5	E	−
Rat			M	+
Wild-mouse/	4	5	E	++
Rat			M	+

−: negative, +: positive, ++: heavily, E: epithelium, M: lamina propria.

surrounding the epithelial buds incorporate androgens but that the epithelium remains receptor negative (see also [13]). This is in contrast to previous findings on androgen receptors in the developing rat prostate gland [5]. These showed receptor positive mesenchyme surrounded receptor negative epithelium during fetal and early postnatal stages while epithelial receptors appeared and mesenchyme ones declined from day 10 postnatal coinciding with the beginning of cytodifferentiation. The results suggested that androgen-activated mesenchyme induced bud formation and that epithelial androgen stimulates cytodifferentiation. The present results do not bear out this interpretation. The fine structure and enzymatic activity of the receptor negative *Tfm* epithelium approximate that seen in the sinus epithelium from normal mouse embryos and it is possible that epithelial androgen is not required for its cytodifferentiation. However, the result does not necessarily deny that the *Tfm* epithelium does not express some androgen-dependent proteins which the normal prostatic epithelium expresses [14].

The exact function of epithelial androgen is still uncertain. It may be a mitogen. In rat and mouse prostate glands in organ culture androgens increased epithelial proliferation in a dose dependent manner [15], but in cell cultures of isolated rat epithelium they did not influence the rate of proliferation [16]. On the other hand, the hormone may be involved in the production of functional substances specific for the fully developed prostate gland such as prostatic binding protein [17], prostatic secretion protein [18], prostatic basic protein, probasin [19] or prostatic acid phosphatase [20].

In addition to the formation of prostate glands the *Tfm* epithelium associated with the rat mesenchyme developed a small number of coagulating glands and seminal vesicles. The coagulating glands originate from the urogenital sinus and the seminal vesicles from the proximal part of the Wolffian ducts and it seems that androgen receptors in the sinus epithelium or Wolffian ducts are also dispensable for the development of these accessory male sex organs. Finally, the results suggest that the mesenchyme does not induce a synthesis of androgen receptors in the *Tfm* epithelium.

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FIGS. 6 and 7. Histochemistry of alkaline phosphatase activity of the prostatic glands induced in a recombinate of the epithelium of wild-type (Fig. 6) or *Tfm* (Fig. 7) and the rat mesenchyme. Magnification ×760.

FIGS. 8 and 9. Histochemistry of nonspecific esterase activity of the prostatic glands induced in a recombinate of the epithelium of wild-type (Fig. 8) and *Tfm* (Fig. 9) rat mesenchyme. Magnification ×760.

FIGS. 10 and 11. Autoradiographs of explants incubated with [^{3}H]testosterone. Sections were stained with haematoxylin. The exposure period was 3 weeks. The glandular epithelium was induced from the sinus epithelium of a wild-type mouse (Fig. 10) and that of a *Tfm* mouse (Fig. 11) in the presence of the rat mesenchyme that incorporated androgen heavily. Magnification ×1,000.

REFERENCES

1 Lasnitzki, I. and Mizuno, T. (1977) Induction of the rat prostate gland by androgens in organ culture. J. Endocrinol., **74**: 47–55.

2 Lasnitzki, I. and Mizuno, T. (1980) Prostatic induction: Interaction of epithelium and mesenchyme from normal wild-type mice and androgen-insensitive mice with testicular feminization. J. Endocrinol., **85**: 423–428.

3 Shannon, J. M. and Cunha, G. R. (1983) Autoradiographic localization of androgen binding in the developing mouse prostate. Prostate, **4**: 367–373.

4 Takeda, H. and Mizuno, T. (1984) Incorporation des androgènes au moment de la cytodifferénciation de l'épithélium prostatique chez le Rat. C. R. Soc. Biol., **178**: 572–575.

5 Takeda, H., Mizuno, T. and Lasnitzki, I. (1985) Autoradiographic studies of androgen-binding sites in the rat urogenital sinus and postnatal prostate. J. Endocrinol., **104**: 87–92.

6 Lasnitzki, I. (1976) The action of androgens on rat prostate glands in organ culture. In "Organ Culture in Biomedical Research". Ed. by M. Balls and M. A. Monnickendam, Cambridge Univ. Press, Cambridge, pp. 241–256.

7 Trowell, O. A. (1959) The culture of mature organs in a synthetic medium. Exp. Cell Res., **16**: 118–148.

8 Karnovsky, M. J. (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol., **27**: 137A.

9 Luft, J. H. (1961) Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol., **9**: 409–414.

10 McGadey, J. (1979) Tetrazolium reaction. In "Enzyme Histochemistry". Ed. by Z. Lojda, R. Gossrau and T. H. Schiebler, Springer-Verlag, Berlin, pp. 61–63.

11 Davis, B. J. and Ornstein, L. (1959) Simultaneous azo-coupling with 1-naphthyl acetate. In "Enzyme Histochemistry (1979)". Ed. by Z. Lojda, R. Gossrau and T. H. Schiebler, Springer-Verlag, Berlin, pp. 109–110.

12 Stumpf, W. E. and Sar, M. (1975) Autoradiographic techniques for localizing steroid hormones. Methods in Enzymol. **36**: 135–156.

13 Mizuno, T., Takeda, H. and Suematsu, N. (1986) Récepteurs d'androgènes de l'épithélium au cours de l'induction des glandes prostatiques à partir de l'épithélium du sinus urogénital de la Souris Tfm-mutant. C. R. Soc. Biol., **180**: 593–595.

14 Suematsu, N., Takeda, H. and Mizuno, T. (1988) Glandular epithelium induced from urinary bladder epithelium of the adult rat does not show full prostatic cytodifferentiation. Zool. Sci., **5**: 385–395.

15 Lasnitzki, I. (1965) Action and interaction of hormones and 3-methylcholanthrene on the ventral prostate gland of the rat *in vivo*. J. Natl. Cancer Inst., **35**: 339–348.

16 McKeehan, W. L., Adams, P. S. and Rosser, M. P. (1984) Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid; cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. Cancer Res., **44**: 1998–2010.

17 Heyns, W. and De Moor, P. (1977) Prostatic binding protein. A steroid-binding protein secreted by rat prostate. Eur. J. Biochem., **78**: 221–230.

18 Pousette, A., Björk, P., Carlström, K., Forgren, B., Högber, B. and Gustafsson, J.-A. (1981) Influence of sex hormones on prostatic secretion protein, a major protein in rat prostate. Cancer Res., **41**: 688–690.

19 Matsuo, Y., Nishi, N., Muguruma, Y., Yoshitake, Y., Kurata, N. and Wada, F. (1985) Localization of prostatic basic protein ("Probasin") in the rat prostates by use of monoclonal antibody. Biochem. Biophys. Res. Commun., **130**: 293–300.

20 Perrin, P., Clairet, F. and Fleury-Goyon, M. C. (1984) Place des phosphatases acides prostatiques dans le traitement des adénocarcinomes de la prostate. J. Urol., **1**: 19–22.

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Plasma Levels of Androgens in Growing Frogs of *Rana nigromaculata*

Sumiko Tanaka, Hisaaki Iwasawa[1] and Katsumi Wakabayashi[2]

Department of Pathology, Kagawa Medical School, Kagawa 761-07,
[1]Faculty of Science, Niigata University, Niigata 950-21, and
[2]Institute of Endocrinology, Gunma University,
Maebashi 371, Japan

ABSTRACT—Using young, subadult and adult *Rana nigromaculata* of both sexes, plasma testosterone and dihydrotestosterone (DHT) levels were determined by radioimmunoassay. In young male frogs, the testosterone concentration was almost constant at ca. 100 pg/ml from just after metamorphosis to July of the next year, while the DHT concentration showed a gradual upward tendency and reached a level twice that of newly metamorphosed frogs by May of the next year. In first-year female frogs, the concentrations of testosterone and DHT were ca. 100 pg/ml, and had doubled by June of the next year. In second-year male frogs, testosterone and DHT concentrations increased remarkably in autumn, especially in DHT, and reached 8.7 ng/ml. In second-year females, testosterone also increased from July on, and reached 1.1 ng/ml in the breeding season (the first half of May) of the following year, though the DHT level remained low. Androgen concentrations in adult males were remarkably low just after hibernation compared with those in the preceding autumn (especially in DHT), decreased further until June, and increased again from July until October as the same manner in second-year males. Testosterone concentrations in adult females showed similar changes, though always slightly lower than those of the male frogs of the same age.

INTRODUCTION

The development of radioimmunoassay (RIA) has enabled one to determine quantitatively very small amounts of hormones in blood. In adult frogs of some anuran species, seasonal changes in the levels of circulating sex hormones have been reported [1–3]. On the other hand, plasma androgen levels in young and subadult frogs have been poorly documented [4]. It is, therefore, difficult to discuss in detail the correlation between plasma androgen concentration and the development of genital tracts, or spermatogenic progress. The present paper describes the changes in the plasma levels of testosterone and DHT in young, subadult and adult frogs of *Rana nigromaculata*, and discusses the relationship between androgen levels and the development of sexual organs in this species [4–8].

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MATERIALS AND METHODS

Animals and serum samples Young, subadult, and adult frogs of *Rana nigromaculata* were collected monthly from May to October, provided that subadult and adult female frogs were to September, at Kanazuka, Niigata Prefecture. The animals collected each time were divided into 6 groups according to sex and age, i.e., first-year (young), second-year (subadult), and third-year or more aged adult males and females. The body length and weight of these frogs were determined, and animals within the range of standard deviation were used as materials. Blood samples were collected in microtubules from the heart of frogs anesthetized weakly by immersion in a 1% solution of MS-222, the blood was centrifuged at 15,000 *g* for 5 min at 4°C. The separated sera were stored at −80°C. Each time serum samples for RIA were made from 6 points. The number of frogs used each point was 6–10 young frogs, 2–4 subadults, and one adult.

Separation of testosterone and DHT After the addition of 1,000 cpm of ^{3}H-testosterone and ^{3}H-DHT, 200 μl of the serum was extracted twice with 4 ml of diethylether. After evaporation of the solvent, the extract was redissolved in 200 μl of an isooctane-benzene-methanol mixture (90:5:5). Testosterone and DHT were then chromatographed on Sephadex LH-20 (Pharmacia Fine Chemicals). One half of the testosterone or DHT fraction was used for assay and the other half was retained to determine recovery with a liquid scintillation counter.

RIA RIA was carried out by a double antibody method according to the method of Ismail *et al.* [9] with minor modification. For the iodination of testosterone-3-carboxymethyloxime-tyrosinemethylester, the chloramine-T method [10] was used. The assay system consisted of: 1) standard or sample dissolved in 200 μl of 1% BSA borate buffer (pH 8.0), 2) 100 μl of diluted antiserum (1:8000, Eiken Immunochemical Laboratory, Tokyo) in 1% normal rabbit serum with 0.05 M EDTA borate buffer (pH 8.0), and 3) 100 μl of ^{125}I-labeled hormone in 1% BSA borate buffer (pH 8.0). The reaction was carried out at 4°C for 2 days. A goat anti-rabbit gamma-globulin serum was used as the second antibody. The amount of labeled testosterone bound in the precipitate was determined with an automatic gamma counter (Beckman, System 8000).

Statistical analyses The data were treated according to the method of one way analysis of variances, and comparison of means were carried out by Duncan's new multiple range test, Student's t-test or Cochran-Cox test depending on the uniformity of variances.

RESULTS

Plasma testosterone concentrations in young male frogs were nearly unchanged from just after metamorphosis to the spring of the next year, showing a slight rise in the middle of June ($P<0.05$ vs. August in first year). DHT levels showed a tendency to rise from middle July to October in the first year ($P<0.25$, one way analysis of variances), and showed a big variation in May of the next year. The level was high in early June ($P<0.01$ vs. July and August in first year) and then fell down until late June. This seasonal change was highly significant ($P<0.025$, analysis of variances).

In young females, on the other hand, plasma testosterone levels were constant during the first year, and rose in early June in the second year ($P<0.05$ vs. first year September and October). Plasma DHT levels in females showed a gradual increase from May to middle June in the second year, then fell down sharply. These changes were significant ($P<0.05$) when examined by the analysis of variances. The mean level in the middle June was significantly higher than those in the first year August and October ($P<0.05$) and than that in middle July in the second year ($P<0.01$) (Fig. 1).

In second-year male frogs, both testosterone and DHT showed a similar fluctuation pattern. That is, they clearly increased in August ($P<0.1$ and $P<0.01$ vs. July, respectively), the concentration of testosterone was ca. 0.5 ng/ml and DHT was ca. 1ng/ml, decreased temporarily in September ($P<0.1$ and $P<0.05$ vs. August, respectively), and then increased markedly in October to 2.5 ng/ml of testosterone and 8.7 ng/ml of DHT. This rise in October was especially remarkable in DHT; the level of DHT was three times higher than that of testosterone.

In adult male frogs, high testosterone levels were found in May, but noticeable individual differences were seen. Then testosterone and DHT concentrations decreased until the middle of June, and increased again from July ($P<0.1$ and $P<0.01$ middle June vs. September, respectively), about a monthe earlier than in subadult male frogs. The concentrations of testosterone and DHT decreased ($P<0.1$ and $P<0.05$ vs. August, respectively) briefly in September and increased strikingly in October. As in the case of second-year male frogs, the concentrations of DHT in the summer and autumn months were always greater than those of testosterone.

In second-year females, the DHT concentration remained at a certain low level from July on. On the other hand, the concentration of testosterone gradually increased from July to September ($P<0.001$ July vs. September, $P<0.01$ July vs August),

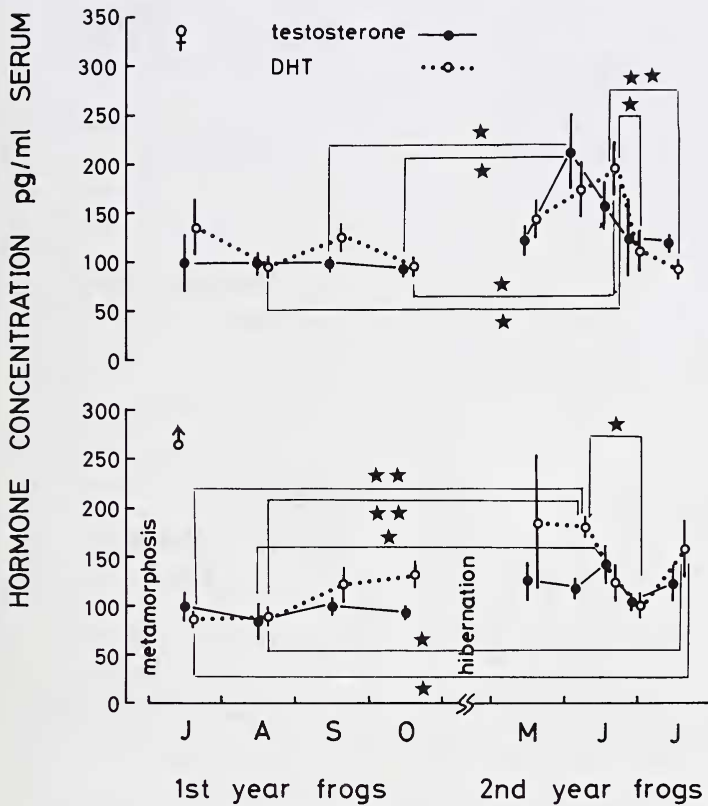

FIG. 1. Concentrations of plasma testosterone and DHT in the frogs from metamorphosis to just before the appearance of secondary sexual characters. Vertical lines indicate S.E. The relation between the two is *: P<0.05 and **: P<0.01.

and reached 1.1 ng/ml in adult female frogs just after hibernation. The level of testosterone was at its minimum after the breeding season ($P<0.05$ May vs. middle June), and increased again after July on ($P<0.01$ middle June vs. September). In subadult and adult female frogs, unlike the male frogs of the same age, no decrease in androgen was seen in September (Fig. 2).

DISCUSSION

Gonadotropins and plasma androgen levels in male frogs

As in the reproductive endocrine system in other vertebrates, a close relationship exists between gonadotropin secretion and androgen production in anurans [11–14]. Licht *et al.* [3] reported that in male *Rana catesbeiana* the concentrations of circulating LH and androgen fluctuated almost in parallel. From the ultrastrucutural quantitative data on gonadotropin-producing cells in subadult male frogs of *R. nigromaculata* [7], gonadotropin control of androgen production is clearly suggested. That is to say, the changes in the number, size, and ultrastructure of gonadotropin-producing cells coincide well with the fluctuation in plasma levels of androgens, especially in the DHT observed in the present study.

Plasma androgen level and spermatogenesis

In newly metamorphosed frogs of *R. nigromaculata*, the seminiferous tubules are almost filled with primary spermatogonia. On the other hand, in young summer and autumn frogs, fairly active spermatogenesis is seen in some parts of the semi-

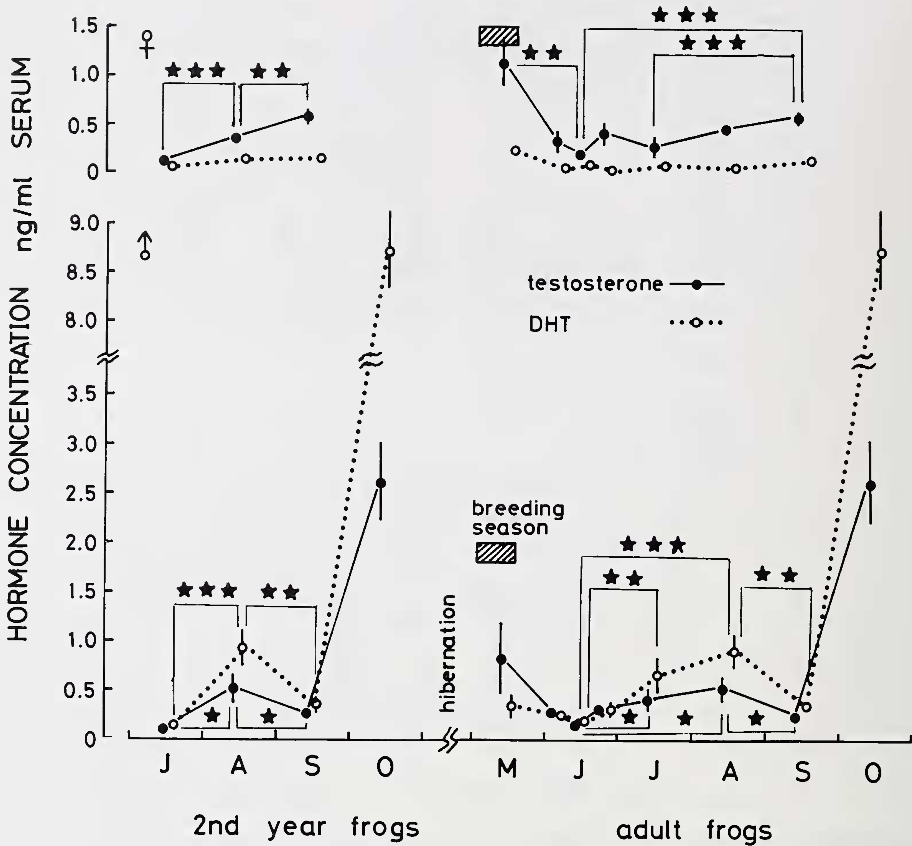

Fig. 2. Concentrations of plasma testosterone and DHT in subadult (2nd-year) and adult frogs. Vertical lines indicate S.E. The relation between the two is *: P<0.1, **: P<0.05 and ***: P<0.01.

niferous tubules, and most of these spermatogenic cells disappear during hibernation [6, 8, 15]. Judging from the results of the present study, it seems that this uneffective spermatogenesis is caused by the deficiency of androgens. Effective spermatogenesis begins in late spring in second-year frogs. In August, in which a remarkable increase in the number of spermatids and spermatozoa is seen, androgen concentrations increased remarkably, and a further increase was seen in October. The thumb pads begin to develop in August, and develop highly in October in this species [4, 5]. Therefore, the progress of spermatogenesis and the development of thumb pads well agree with the increase in the concentration of plasma androgens. The reason for the decrease in androgen seen in September is unknown.

A wide variation in the concentration of plasma testosterone was seen in adult male frogs of the breeding season. This phenomenon may be due to the use of frogs having different breeding activities, and it seems that the testosterone concentration rapidly decreases toward the end of the breeding season. It is interesting that the available androgen in the breeding period is testosterone rather than DHT.

The changes in the plasma androgen concentration observed in the present study coincided with the results of Kera and Iwasawa [4], except for the values in second-year May frogs, and the reason for this disagreement is not known.

Plasma androgen level and development of genital tracts

Sexual difference in genital tracts in *R. nigromaculata* appeared rapidly and remarkably in second-